See Colour Plate 114.

See also: **II / Pigments:** Thin-Layer (Planar) Chromatography. **Terpenoids: Liquid Chromatography.**

Further Reading

Garrido JL and Zapata M (1996) Ion-pair reversed-phase high-performance liquid chromatography of algal chlorophylls. *Journal of Chromatography A* 738: 285.

Hodgson DA, Wright SW and Davies N (1997) Mass spectrometry and reverse phase HPLC techniques for the identification of degraded fossil pigments in lake sediments and their application in palaeolimnology. *Journal of Paleolimnology* 18: 335.

Jeffrey SW, Mantoura RFC and Wright SW (eds) (1997) *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*. Paris: UNESCO.

Mantoura RFC and Llewellyn CA (1983) The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. *Analytica Chimica Acta* 151: 297.

Millie DF, Paerl HW and Hurley JP (1993) Microalgal pigment assessments using high-performance liquid chromatography: a synopsis of organismal and ecological applications. *Canadian Journal of Fisheries and Aquatic Sciences* 50: 2513.

Roy S (1987) High-performance liquid chromatographic analysis of chloropigments. *Journal of Chromatography* 391: 19.

Šestak Z (1982) Thin layer chromatography of chlorophylls 2. *Photosynthetica* 16: 568.

Van Breemen RB (1996) Innovations in carotenoid analysis. *Analytical Chemistry* 68: 299A.

Van Heukelem L, Lewitus AJ, Kana TM and Craft NE (1994) Improved separations of phytoplankton pigments using temperature-controlled high performance liquid chromatography. *Marine Ecology Progress Series* 114: 303.

Wright SW, Jeffrey SW, Mantoura FRC *et al*. (1991) Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series* 77: 183.

Thin-Layer (Planar) Chromatography

G. W. Francis, University of Bergen, Bergen Norway

Copyright © 2000 Academic Press

DropBooks

General Introduction

Colour and Pigments

The colours found in natural organisms are of two types: those which are due to structural effects, and those which result from the presence of pigments. The two types of colouring often occur together. Examples of structural colour are to be found in the scales of fish, the wings of butterflies and the hair of many animals, although structural colour is also to be found in the plant world. The apparent colour depends on the interplay of these effects. It is thus important to realize at the outset that only the colour due to the pigments can be extracted. However, even then the *in vivo* and *in vitro* colours can be vastly different, since the pigments themselves show different colours according to the environment in which they occur – whether in fat globules or aqueous complexes.

Importance

Colour is used by almost all organisms to communicate in one form or another, for example, the defensive colours of insects, the attractive colours of flowers and fruit. The way in which various organisms produce pigments is genetically determined, and thus related organisms have similar pigment patterns and biologists use pigments to classify organisms at all levels. The commercial value of agricultural and particularly horticultural products is often closely related to their colour.

Natural Pigment Structures

The number of main pigment groups is relatively small. While various pigment groups are of vastly different skeletal types, there is usually little variation of the skeleton within each group and in many cases the possible group of pigments involved can be arrived at based on the observed colour in nature. Thus, while the green colour of chlorophylls is immediately recognizable, the typical flavonoids provide pale yellow colours, and the anthocyanidins, a special group of flavonoid salts, are usually responsible for red-blue colours in plants. The deep yellow to orange red shades resulting from the presence of carotenoids are also readily identified. Variations within a particular group are usually due to two factors: the degree of oxidation and the presence of substituents.

Extraction Methods

The different types of pigment vary greatly in polarity and in their sensitivity to chemical reagents, and thus require different extraction methods. It is best to avoid the use of acids or bases unless necessary, and

as far as possible exposure to air or light. It should be noted that working rapidly and with normal care may sometimes give better results than working with time-consuming rigorous methods, at least at a qualitative level. Normally, a solvent is found that will extract the required pigment type while extracting as little as possible of the remaining components from the substrate. This means essentially that the solvent and pigment type should match as well as possible with respect to polarity. However, even such tailored methods can fail if unusual or unsuspected substituents are present and lead to the identification of artefacts as naturally occurring.

Clean-up and Pretreatment

Even when extracts or individual pigments appear to be pure, the undisclosed presence of colourless impurities should always be investigated. Such impurities are normally readily disclosed by spraying a trial chromatogram with a strong oxidizing agent. This often provides valuable information before final chromatography is undertaken, since the presence of such impurities greatly reduces the efficiency of the plates, in terms of both resolution and capacity. The most common clean-up treatment is the extraction of polar impurities from less polar extracts, or the reverse where waxes are removed either before or after the extraction process. It is important to ensure the removal of residual solvents after such clean-up procedures, or indeed after saponification or hydrolysis, as they will otherwise result in inferior separation. Residual solvents are a common source of problems in chromatography and, in particular, the presence of even minor traces of water (easily removed by azeotropic distillation) can often spoil the separation of hydrophobic pigments.

Chromatography Layers

Chromatography may be carried out on layers coated on plastic or aluminium foils, or glass plates. The type used depends to some extent on whether a quantitative analysis is required since the backing is critical for densitometric methods. Commercial pre-coated plates have the advantage of uniform quality and give more reproducible results than laboratory-coated plates. The procedures given below have all been carried out on commercial plates for this reason. However, it should be noted that only a few adsorbents are available and the plates are expensive if large numbers are required. In addition, some commercial plates contain compounds used in their manufacture which may be extracted if the plates are used preparatively. It is normally possible, with practice, to produce laboratory-made plates that give similar results, and in this case there are almost unlimited possibilities for mixing different adsorbents. In the case of larger scale preparative work, it is often easier to work with less dense home-made layers, although care should be taken in handling them to avoid damaging the layer.

Quantification

Densitometry is readily applied to the measurement of pigments, provided the usual precautions are taken to choose suitable wavelength, and drying of the developed plate is carried out in a manner that avoids excessive decomposition. Since pigments are usually worked with on small scales, thin layers are well suited for their preparative or semipreparative separation.

Pigment Groups

The pigment groups treated in more detail below are the main photosynthetic pigments, carotenoids and chlorophylls, and the most widely distributed flavonoid classes, with a separate section for the anthocyanins. Finally, other pigment types are discussed briefly.

Carotenoids

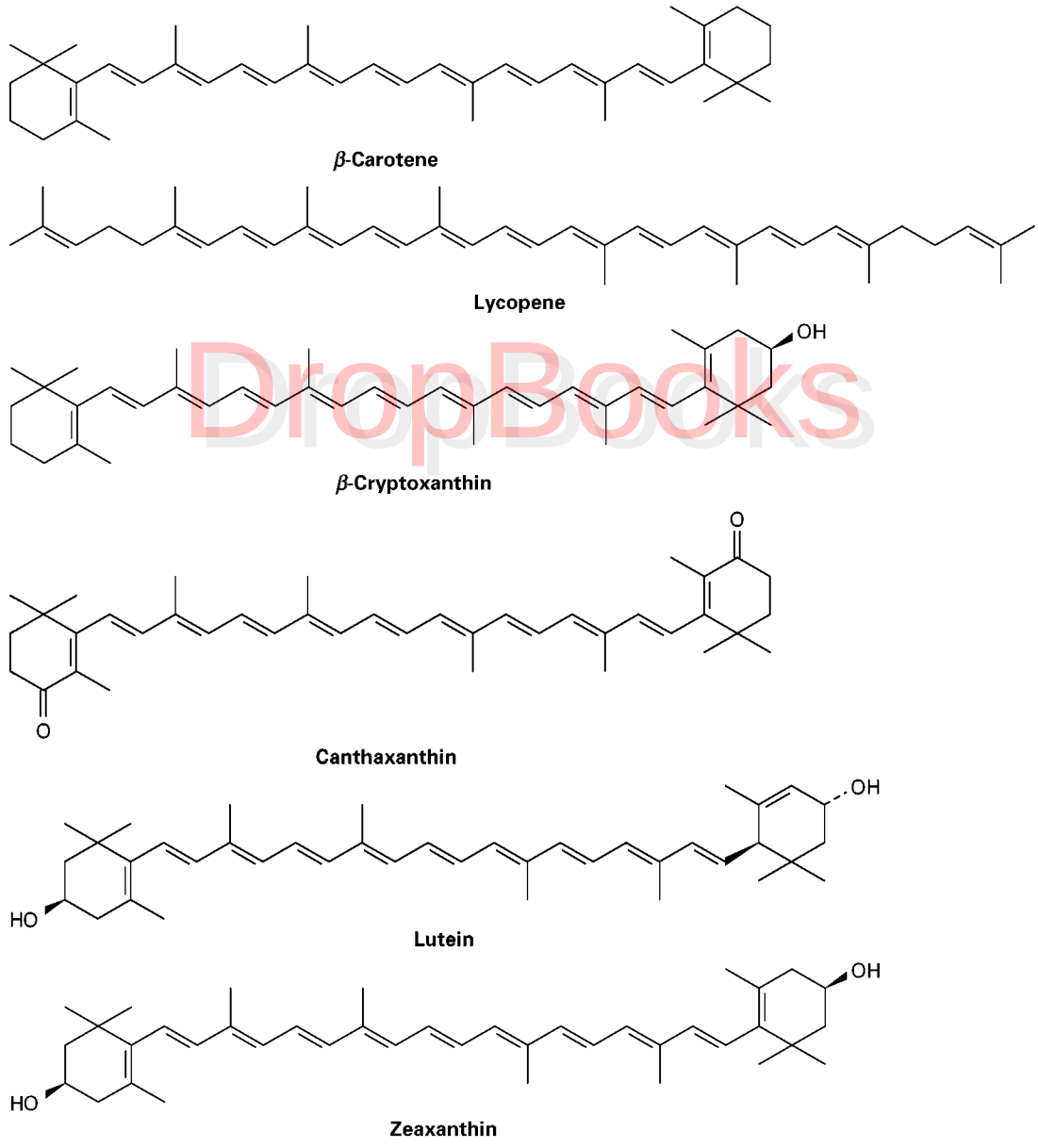
The carotenoids are probably the most widely distributed group of pigments, occurring in all photosynthetic organisms, in most animals and in a wide variety of microorganisms. They normally occur in the free form or as fatty acid esters, although carotenoproteins are common in marine animals and glycosides are found in some microorganisms. They are usually yellow to red in colour, although carotenoproteins are able to provide a full range of colours.

The carotenoids are tetraterpenoids and may be thought of as being made up by a central conjugated chain carrying two end-groups. Some typical carotenoids are shown in **Figure 1**. The number of possible end-group skeletons is quite restricted and structural variation largely derives from the type and position of the functional groups which they contain. The compounds containing only carbon and hydrogen are often called carotenes to differentiate them from their oxygenated analogues, which are then designated as xanthophylls. While all of the compounds in Figure 1 have 40 carbon atoms, carotenoids containing partial skeletons are known, as are compounds carrying extra isoprenoid units to give skeletons having 45 and 50 carbon atoms.

Extraction methods for carotenoids depend somewhat on the organism being examined. Tissues from higher plants and microorganisms, where carotenoids

are usually present in the free form or as esters, can usually be extracted by hydrocarbon–acetone mixtures. Problems may be encountered with some microorganisms where it is necessary to disrupt the cells before extraction, where compounds may be strongly bound to cell wall material or, less often, where more polar solvents are required to extract glycosidic carotenoids. Animal tissues can contain carotenoproteins and in such cases a choice must be made between extraction with acetone which will free the hopefully unchanged carotenoids from the protein or extraction with suitable buffers of the intact carotenoproteins from which the carotenoids can then be liberated.

The normal procedure for carotenoid analysis is to examine the initial extract to decide whether the compounds are present in the free form or as esters. The esters themselves are much less polar than the free xanthophylls and a check can be made by saponifying an aliquot of the extract. Given a positive result a saponification step can then be applied to the whole extract, although it should be noted that some few carotenoids may suffer changes, e.g. astaxanthin, the main pigment in salmonoids, is changed to the closely related astacene by base treatment. One advantage of saponification of plant extracts is that the chlorophylls, which will otherwise interfere with chromatography, are destroyed and can be easily



DropBooks

Figure 1 Structures of some typical carotenoids.

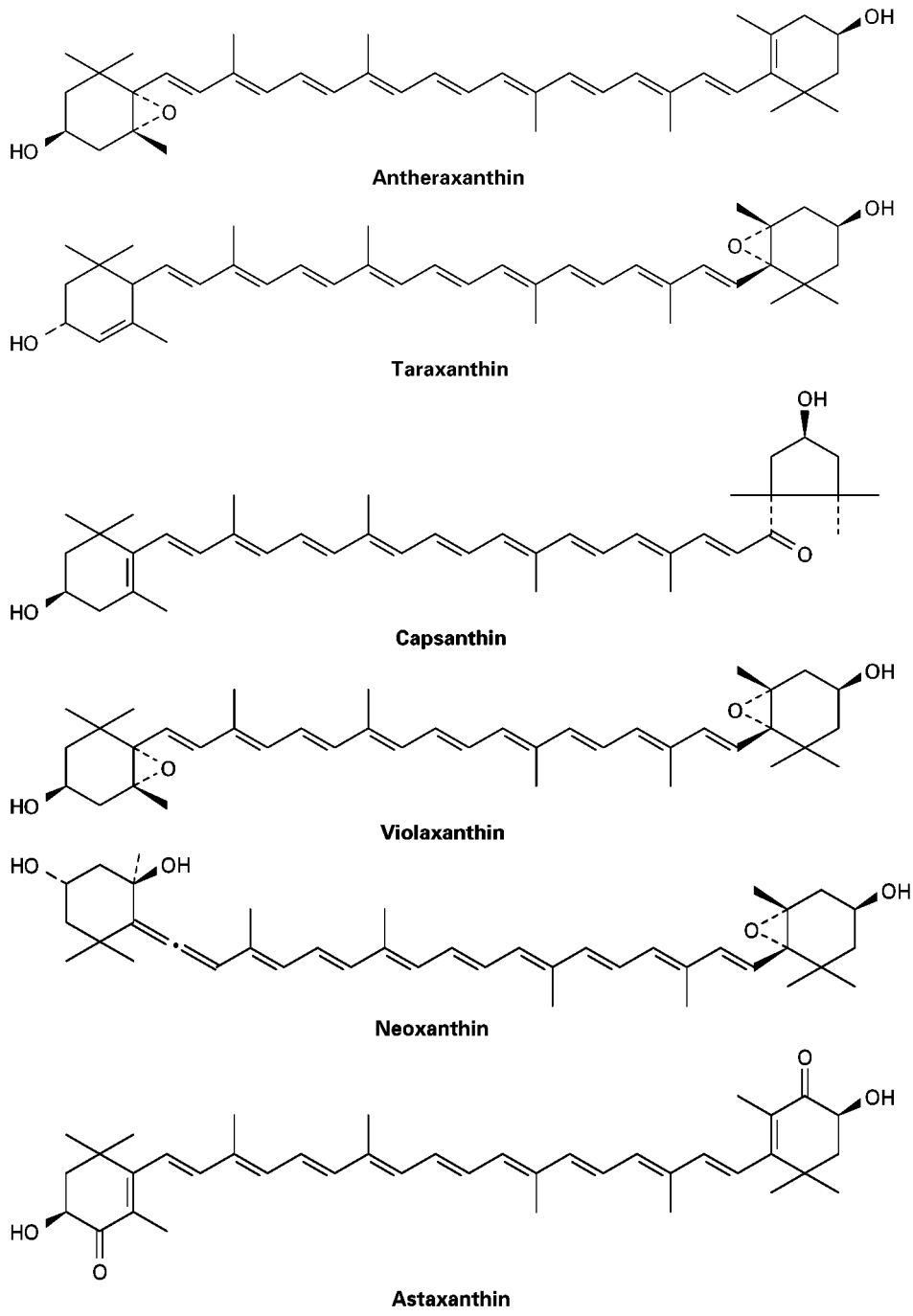


Figure 1 *Continued.*

removed prior to analysis. The effects of saponification can readily be seen in **Figure 2**, which shows a chromatogram of a paprika extract before and after saponification. The unsaponified extract is dominated by the presence of esterified carotenoids, while the esters are totally absent after saponification.

The finished saponified extract should be applied to the plate in a volatile solvent, usually acetone or diethyl ether. While a large number of stationary phases have been employed, the most reliable are silica gel for normal-phase and octadecylsilylsilica for reversed-phase work, and these suffice to separate the normal range of pigments. If only carotenes are to be analysed, laboratory-coated magnesium oxide plates can be employed. R_F values are given for some common carotenoids in **Table 1**, for both normal-phase and reversed-phase systems. These values show immediately that retention is affected by the number

Figure 2 Chromatography of paprika (*Capsicum annuum*). Silica gel 60 (0.25 mm, Merck, Art. 5721) 40% acetone–petroleum ether. (A) Before saponification; (B) after saponification. Identification of zones: 1, β-carotene; 2, capsanthin diesters; 3, capsorubin diesters, 4, zeaxanthin and other diol diesters; 5, capsanthin monoesters; 6, capsorubin monoesters; 7, capsanthin; 8, cryptoxanthin, 9, zeaxanthin; 10, violaxanthin; 11, capsorubin, 12, neoxanthin. Only main zones are shown: filled zones indicate red colour; other zones are yellow.

and nature of functional groups present. Hydroxyl functions have greater effects than carbonyls, which in turn have greater effects than ether groups. Longer chains are more retained than shorter ones in normal-phase chromatography, while the opposite is true for reversed-phase systems. It should be noted that, while development on silica gel layers with 40% acetone–petroleum ether (40–60°C) gives a generally similar result to that obtained with 20% of *tert*-butanol or *tert*-pentanol, carbonyls have markedly more retention in the latter systems. This difference in behaviour can be utilized when separating complicated mixtures of closely related carotenoids. The choice as to whether to use normal or reversed-phase plates will depend on the compounds present.

Practical procedures All operations with carotenoids should be carried out in dim light, avoiding exposure to air and acids and at temperatures not exceeding 40°C. Carotenoid samples and extracts should be kept in the refrigerator as far as possible.

Extraction The material to be extracted is cut into suitable pieces (1 cm cubes) suspended in three volumes of acetone and subjected to maceration for 2–3 min in a laboratory mixer. The solvent is removed and retained, and the procedure repeated twice. At this stage, much of the water present in the original tissue has been extracted and it is often advantageous to carry out one or two further extractions with acetone–pertoleum ether (40–60°C) in a 1 : 1 v/v ratio. Where very polar carotenoids are present, extraction with solvent mixture containing methanol may be required. When the residue is colourless, the extracts are pooled and taken to dryness under reduced pressure. The extract can then be tested for the presence of esters and, in their absence, applied to the thin layers as solutions in diethyl ether or acetone. Otherwise saponification should be performed.

Saponification The dried extract is dissolved in a small amount of diethyl ether or methanol to give a deeply coloured concentrated solution which is then diluted with several volumes of diethyl ether. Saponification is then ensured by adding an equal volume of 10% methanolic sodium hydroxide, replacing the air in the flask with nitrogen, closing firmly, and after brief shaking allowing the mixture to stand at room temperature for 6 h in the dark. After this time the saponification mixture is diluted to three times its volume with 5% aqueous sodium chloride. The carotenoids are then extracted with similar volumes of diethyl ether until the diethyl ether extract is no longer coloured. The combined diethyl ether extracts are then pooled and washed to neutrality with succesive portions (normally at least three) of 5% aqueous sodium chloride, before a final wash with

DropBooks

Table 1 Thin-layer chromatography of carotenoids: R_β values (β-carotene = 1.00) for systems 1–4, and R_F values for systems 5 and 6

Carotenoid	*Source*	*Chromatographic system*					
		1	*2*	*3*	*4*	*5*	*6*
β-Carotene	a	1.00	1.00	1.00	1.00	0.10	0.13
Lycopene	b	1.00	0.97	1.00	1.00	0.17	0.23
β-Cryptoxanthin	a	0.72	0.34	0.78	0.76	0.22	0.31
Canthaxanthin	c	0.71	0.36	0.69	0.66	0.26	0.38
Lutein	d	0.44	0.07	0.56	0.53	0.37	0.55
Zeaxanthin	e	0.44	0.07	0.55	0.50	0.37	0.57
Antheraxanthin	f	0.40		0.40	0.31	0.44	0.60
Taraxanthin	e	0.41		0.43	0.37	0.47	0.62
Capsanthin	g	0.39		0.38	0.28	0.48	0.64
Violaxanthin	d	0.33		0.30	0.19	0.55	0.68
Neoxanthin	d	0.18		0.13	0.08	0.63	0.72
Astaxanthin	h	0.09		0.07		0.85	0.91

System 1: Silica gel 60 (0.25 mm, Merck, Art. 5721) 40% acetone–p.e.; system 2: silica gel 60, 20% acetone–p.e.; system 3: silica gel 60, 20% *tert*-pentanol–p.e.; system 4: silica gel 60, 20% *tert*-pentanol–p.e.; system 5: RP-18 F_{254} (0.25 mm, Merck, art. 15425) p.e.–acetonitrile–methanol (10 : 40 : 50); system 6: RP-18 F_{254}, p.e.–acetonitrile–methanol (20 : 40 : 40). Solvent compositions by volume; p.e., petroleum ether (40–60°C). Sources: a, *Sorbus aucuparia* berries; b, *Solanum lycopersicum* fruit; c, commercial; d, *Petroselinum crispum*; e, *Taraxacum officinale* flowers; f, *Lilium x hollandicum* flowers; g, *Capsicum annuum* fruit; h, *Salmo salar*.

water. The washed extract is taken to dryness under reduced pressure and dissolved in either acetone or diethyl ether for analysis.

Chromatography The best initial approach to the separation of the carotenoid mixture is to carry out trial runs on silica gel layers using as developers increasing amounts of acetone in petroleum ether (40–60°C), e.g. 0, 5, 10, 20 and 40% v/v. This will immediately give an indication of the polarity of the compounds present and provide guidelines for further work. Where separations are reasonable but there is overlap, a new chromatogram based on the best acetone percentage should be developed with approximately half of that percentage of *tert*-butanol. If normal-phase methods fail or if there is a preponderance of polar compounds, reversed-phase separation should be tried; possible systems are indicated in Table 1. Where single development is insufficient to provide separation, the various zones can usually be scraped off the plate and re-extracted with good recovery rates provided that the process is done quickly and with due care. Separation within the various zones can then be carried out by renewed chromatography with appropriate solvent mixtures, i.e. solvents giving R_F values of about 0.50.

Identification Relatively few carotenoid standards are available commercially, but a large number of natural sources are well documented, and the monographs of Goodwin from 1980 and 1984 are particularly useful sources for this information. Otherwise, many carotenoids can be identified even in small quantities from their visible light absorption spectra and by microreactions.

Chlorophylls

The chlorophylls are essential for photosynthesis and are thus of universal distribution in photosynthetic organisms. They are tetrapyrroles with a cyclic structure, and in intact tissue occur coordinated to a magnesium (2+) ion. The main pigments, chlorophylls *a* and *b*, differ only in having one methyl substituent in chlorophyll *a*, replaced by a formyl group in chlorophyll *b*. The intact chlorophylls also contain a phytyl group which confers a hydrophobic moiety on an otherwise hydrophillic structure (see **Figure 3** for structures for chlorophylls *a* and *b*). Chlorophylls *a* and *b*, which absorb light in both blue and red spectral areas, are green in colour, the former being described as blue-green and the latter as yellow-green.

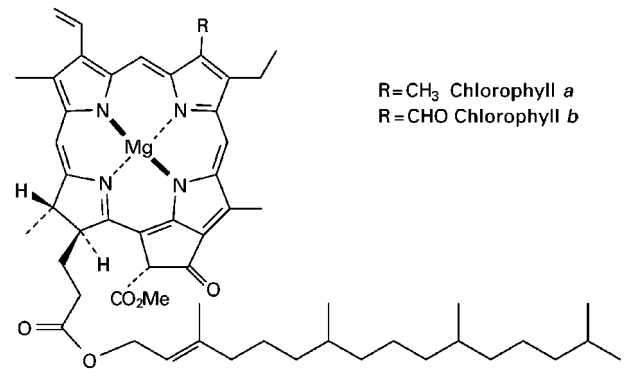


Figure 3 Structure of chlorophylls.

Table 2 Thin-layer chromatography of chlorophylls and derivatives (R_F values)

Chlorophyll derivative	R	R′	*Magnesium*	*Colour*	*System 1*	*System 2*
Pheophytin *a*	CH_3	Phy	No	Grey	0.93	0.40
Pheophytin *b*	CHO	Phy	No	Yellow-brown	0.88	0.33
Chlorophyll *a*′	CH_3	Phy	Yes	Blue-green	0.80	0.31
Chlorophyll *a*	CH_3	Phy	Yes	Blue-green	0.76	0.27
Chlorophyll *b*′	CHO	Phy	Yes	Yellow-green	0.60	0.25
Chlorophyll *b*	CHO	Phy	Yes	Yellow-green	0.57	0.22
Pheophorbide *a*	CH_3	H	No	Grey	0.36	
Pheophorbide *b*	CHO	H	No	Yellow-brown	0.18	
Chlorophyllide *a*	CH_3	H	Yes	Blue-green	0.08	
Chlorophyllide *b*	CHO	H	Yes	Yellow-green	0.05	

Structures may be derived from Figure 3 (Phy = phytyl). System 1: cellulose layer (0.1 mm, Merck Art. 5716), light petroleum (40–60°C)–acetone (80 : 20). System 2: silica gel 60 (0.25 mm, Merck Art. 5721), diethyl ether–acetone–isooctane (20 : 20 : 60). All compounds show red fluorescence under UV light.

The relative amounts of chlorophylls *a* and *b* found in higher plants vary according to the species being investigated and prevailing light conditions, a ratio of about 3 : 1 being normal, while the ratio increases in plants growing in sunny situations and decreases in those found in shade. The C-10 epimers, chlorophylls *a*′ and *b*′, which co-occur with the main chlorophylls in extracts, are now believed to be present in intact tissue. Other compounds often found in extracts are pheophytins *a* and *b* which differ from the parent chlorophylls only in lacking the magnesium ion, and the pheophorbides where the phytyl chain too has been lost. In addition, the very polar chlorophyllides which are simply derived by hydrolysis of the phytyl ester function in chlorophylls *a* and *b* are often present in small amounts. The pheophytins, pheophorbides and chlorophyllides are regarded as decomposition products produced during extraction.

Water-miscible solvents such as methanol and acetone are normally used for extraction of the chlorophylls which is often carried out in the presence of sodium carbonate to ensure neutralization of acidic impurities. Great care is also required during extraction to avoid undue exposure to heat or light as these lead to increased structural alteration. The extracted pigments are transferred to diethyl ether for concentration and analysis. Chlorophylls can be purified as their readily formed and moderately stable dioxane complexes.

Chlorophylls are normally applied to either cellulose or silica gel thin layers as acetone or diethyl ether solutions. Retention behaviour for illustrative systems may be found in **Table 2**, where structural parameters are also indicated. A densitometric trace obtained after chromatography of parsley chlorophylls can be found in **Figure 4**. In all cases the chlorophyll *a* derivative is less retained than the *b* derivative, an expected result of the presence of the additional aldehyde function in the latter set. While loss of the magnesium ion leads to a reduction in polarity, loss of the phytyl group has the opposite effect. The chlorophyllides which lack the lipophilic phytyl chain remain close to the baseline in these systems.

Extraction The tissue being examined is mixed with 5 volumes of acetone containing small amounts of sodium carbonate, and macerated in a laboratory mixer for 2–3 min. This procedure is repeated until the matrix is colourless. The extracts are combined and concentrated to small volume under reduced pressure, 5% aqueous sodium chloride is added (to increase ionic strength and lessen the solubility of the pigments in the aqueous phase) and the resulting mixture is ex-

DropBooks

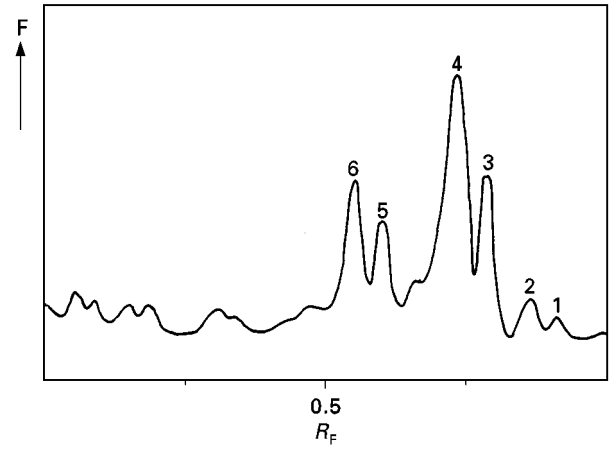


Figure 4 Densitometric trace (fluorescence, excitation at 366 nm, reflectance mode) of a chromatogram of chlorophyll pigments and derivatives obtained from parsley (*Petroselinum crispum*). Cellulose layer (0.1 mm, Merck Art. 5716), light petroleum (40–60°C)–acetone (80 : 20). Identification of zones: 1, pheophytin a; 2, pheophytin b; 3, chlorophyll *a*′; 4, chlorophyll *a*; 5, chlorophyll *b*′; 6, chlorophyll *b*. Minor zones are not identified.

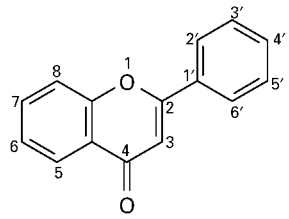


Figure 5 Flavone skeleton showing standard numbering.

tracted repeatedly with similar volumes of diethyl ether. The diethyl ether extracts are combined and taken to dryness under reduced pressure at less than 40°C. The dried material can now be dissolved in fresh diethyl ether for application to the thin-layer plates.

Identification All chlorophyll derivatives show a bright red fluorescence under UV ultraviolet light. Chlorophylls *a* and *b* are commercially available, but can be readily obtained by extraction as described above from spinach. Pheophytins can be prepared by treating diethyl ether solutions of the parent chlorophylls for 2 min with 13% hydrochloric acid. The use of more concentrated acid (30%) leads to the production of the pheophorbides. Chlorophyllides can be obtained if required by enzymatic hydrolysis of the chlorophylls themselves.

Flavonoids

The major classes of the yellow flavonoids are the flavones and flavonols and these will be discussed here. In the next section, the red-blue anthocyanins will be treated. The minor flavonoid classes can in most cases be investigated by similar systems to those described here for flavones and flavonols. The flavonoids are widely distributed in the plant kingdom and are said to occur in all vascular plants. Flavonoids can occur either as the aglycones or more usually as glycosides; the number of known glycosides of quercetin, for example, is in excess of 100.

The flavonoids are made up of two phenyl rings connected by a 3-carbon unit; the various classes are defined by the nature of the bridging unit between the two aromatic systems. The basic flavone structure and the numbering of the skeleton are given in **Figure 5**. Flavonols are regarded as a separate class although they differ from the flavones only in having a 3-hydroxyl group. The normal positions for oxygenation in these compounds are at 5 and 7 in the condensed system (ring A) and at 3′, 4′ and 5′ in ring B.

Flavonoids are readily extracted from plant tissue by maceration in warm methanol and methanol–water mixtures. Where flavonoids are present as glycosides, additional information is to be had by

Table 3 Thin-layer chromatography of flavonoid glycosides (R_F values)

Compound	*Source*	*Position of OH-substituents*	R_F
Flavone glycosides			
Apigenin-7-O-glu	c	5,7,4′	0.57
Apigenin-8-C-glu	a	5,7,4′	0.56
Apigenin-7-O-apiosylglu	d	5,7,4′	0.39
Apigenin-6-C-glu-7-O-glu	b	5,7,4′	0.20
Luteolin-7-O-glu	c	5,7,3′,4′	0.54
Diosmetin-7-O-rhaglu	e	5,7,3′ and 4′-OMe	0.31
Flavonol glycosides			
Kampferol-3-O-rha	f	3,5,7,4′	0.72
Kampferol-3-O-glu	g	3,5,7,4′	0.65
Kampferol-3-O-gal	f	3,5,7,4′	0.59
Quercetin-3-O-rha	h	3,5,7,3′,4′	0.69
Quercetin-3-O-glu	i	3,5,7,3′,4′	0.53
Quercetin-3-O-gal	h	3,5,7,3′,4′	0.51
Quercetin-3-O-rut	j	3,5,7,3′,4′	0.30
Isorhamnetin-3-O-glu	k	3,5,7,4′ and 3′-OMe	0.58
Isorhamnetin-3-O-rut	k	3,5,7,4′ and 3′-OMe	0.36
Myricetin-3-O-rha	l	3,5,7,3′,4′,5′	0.58
Myricetin-3-O-glu	m	3,5,7,3′,4′,5′	0.46
Myricetin-3-O-gal	n	3,5,7,3′,4′,5′	0.45

System: Silica Gel 60 F_{254} (0.25 mm, Merck Art. 5715) with developing solvent: ethyl acetate–formic acid–acetic acid–water (100 : 11 : 11 : 27). Sources: a, *Anthemis nobilis*; b, *Crataegus monogyna*; c, *Petroselinum* spp.; d, *Saponaria officinalis*; e, *Diosma crenulata*; f, *Menyanthes trifoliata*; g, *Astragalus* spp.; h, *Betula* spp.; i, *Equisetum arvense*; j, *Ruta graveolens*; k, *Calendula officinalis*; l, *Myrica rubra*; m, *Primula sinensis*; n, *Camellia sinensis*. glu, glucoside; rut, rutinoside; gal, galactoside; rha, rhamnoside.

subjecting them to acid hydrolysis which only C-glycoside links survive. The products are recovered, dissolved in organic solvent and then subjected to chromatographic analysis.

While a large number of systems can be used to analyse the glycosides, experience suggests that adequate separations can be obtained on silica gel with a solvent systems containing ethyl acetate–formic acid–acetic acid–water in the proportions 100 : 11 : 11 : 27 v/v. Visualization is accomplished by spraying with a 1% diphenylborinic acid ethanolamine ester (Natural Product Reagent A) solution in methanol and thereafter with polyethylene glycol (PEG 4000). The plates are examined under ultraviolet light (366 nm) and the compounds are seen as spots in various yellow-orange-brown-green hues. **Table 3** gives the chromatographic behaviour of some flavonoid glycosides with this system. It is apparent that the fact that the flavonols have an extra hydroxyl function does not give an immediately obvious increase in polarity. However, it should be borne in mind that the glycosidic substituent is found on the less exposed 3-position in the flavonols as against more exposed positions in the flavones. However, within each group it is apparent that increasing oxygenation increases polarity somewhat. Much larger changes in polarity are observed on changing the sugar involved: the rhamnosides are less retained than the glucosides, which in turn are less retained than the galactosides. Unsurprisingly, the biosides and diglycosides are again more retained than the simple monoglycosides.

A wide variety of systems are available for the analysis of free flavonoid aglycones. Examples of such analysis using silica gel, polyamide and reversed-phase (RP 18) layers are given in **Table 4**. The detection system used is as for the glycosides. Here the trends are more readily seen, with increasing oxygenation, in particular the presence of additional hydroxyls, in general leading to increasing polarity. However, the presence of the 3-OH group in the flavonols opens the possibility of hydrogen bonding to the 4-keto group and thus a corresponding reduction in polarity. Correspondingly, while O-methylation normally reduces polarity, this effect is much reduced when the methylation is of the 3-OH group, since this again frees the keto group from intramolecular hydrogen bonding.

Extraction procedure The intact tissue is cut into small pieces and then macerated with 3 volumes of warm methanol for 2 min, and thereafter stirred for a few

DropBooks

Table 4 Thin-layer chromatography of flavonoid aglycones (R_F values)

Pigment	*Colour*	*Substituents*		*System*		
	(*UV*)	*OH*	*OMe*	1	2	3
Flavones						
Flavone	Blue			0.60	0.86	0.29
5-Hydroxyflavone	Brown	5		0.74	0.88	0.22
7-Hydroxyflavone	Brown	7		0.48	0.51	0.41
Chrysin	Brown	5,7		0.57	0.52	0.33
Apigenin	Green	5,7,4′		0.46	0.18	0.48
Acacetin	G-green	5,7	4′	0.54	0.45	0.30
Apigenin-7′,4′-dimethyl ether	G-green	5	7,4′	0.66	0.86	0.14
Luteolin	Yellow	5,7,3′,4′		0.38	0.06	0.53
Diosmetin	Green	5,7,3′	4′	0.43	0.41	0.46
Flavonols						
Kaempferol	Y-green	3,5,7,4′		0.48	0.14	0.57
Kaempferid	B-green	3,5,7	4′	0.59	0.38	0.37
Kaempferol-7,4′-dimethyl ether	B-green	3,5	7,4′	0.73	0.74	0.18
Kaempferol-3,7,4′-trimethylether	Green	5	3,7,4′	0.69	0.87	0.19
Quercetin	Brown-o	3,5,7,3′,4′		0.41	0.06	0.67
Rhamnetin	Orange	3,5,3′,4′	7	0.48	0.24	0.45
Quercetin-3,7-dimethyl ether	Orange	5,3′,4′	3,7	0.46	0.47	0.46
Morin	Green	3,5,7,2′,4′		0.29	0.02	0.73
Fisetin	Orange	3,7,3′,4′		0.36	0.08	0.70
Robinetin	Orange	3,7.3′,4′,5′		0.22	0.01	0.82

System 1: Silica gel 60 F_{254} (0.25 mm, Merck Art. 5715) toluene–ethyl formate–formic acid (50 : 40 : 10); system 2: polyamide (0.15 mm, Merck Art. 5555/0025), toulene–butanone–methanol (60 : 25 : 15); system 3: RP-18 F_{254} (0.25 mm, Merck Art. 15425) methanol–formic acid–water (58 : 10 : 16). Solvent compositions by volume. Colour observed under 366 nm after spraying with Natural Product Reagent A followed by polyethyleneglycol (PEG-4000): b, blue; o, orange; y, yellow.

minutes while heating to about 60°C. Filtration can be followed by a second extraction with a 1 : 1 mixture of methanol and water. The extracts are combined, the solvents removed and the pigments redissolved in methanol prior to application to the thin layers for separation.

Hydrolysis The dried extract is dissolved in equal volumes of methanol and 2 mol L^{-1} hydrochloric acid and refluxed for 1 h. The mixture is then cooled and the aglycones extracted into diethyl ether or ethyl acetate. The resultant solution is taken to dryness under reduced pressure and then redissolved in fresh organic solvent prior to analysis. The sugars freed during this process remain in the water solution and these too may be analysed by thin-layer methods.

Identification A considerable number of flavonoid aglycones and glycosides are available commercially. Pure compounds can be isolated from established sources, many of which can be found in the monographs on flavonoid compounds given in the Further Reading section. In the absence of reference compounds, observed polarities and the colours in ultraviolet light after treatment with diphenylboronic acid ethanolamine ester provide valuable evidence of identity.

Anthocyanins

The glycosidic anthocyanins are closely related to the flavonoid classes discussed above, sharing the same basic skeleton. They lack the 4-keto function and have the heterocyclic ring aromatized and thus occur as salts. They provide red-blue colours depending on the substitution pattern present. Only six aglycone anthocyanidins are usual and the structures of these are to be found in **Figure 6**. Both glycosides and aglycones are found in red to purple plant tissues in plants, and they are responsible for the familiar colours of many deep red fruits and fruit juices and red wine, as well as occurring in the leaves of many red-purple ornamental varieties of trees and bushes.

Since the anthocyanins are salts they must be extracted into acidic media. Methanol containing 1% concentrated hydrochloric acid is well suited, although if milder conditions are required, as for

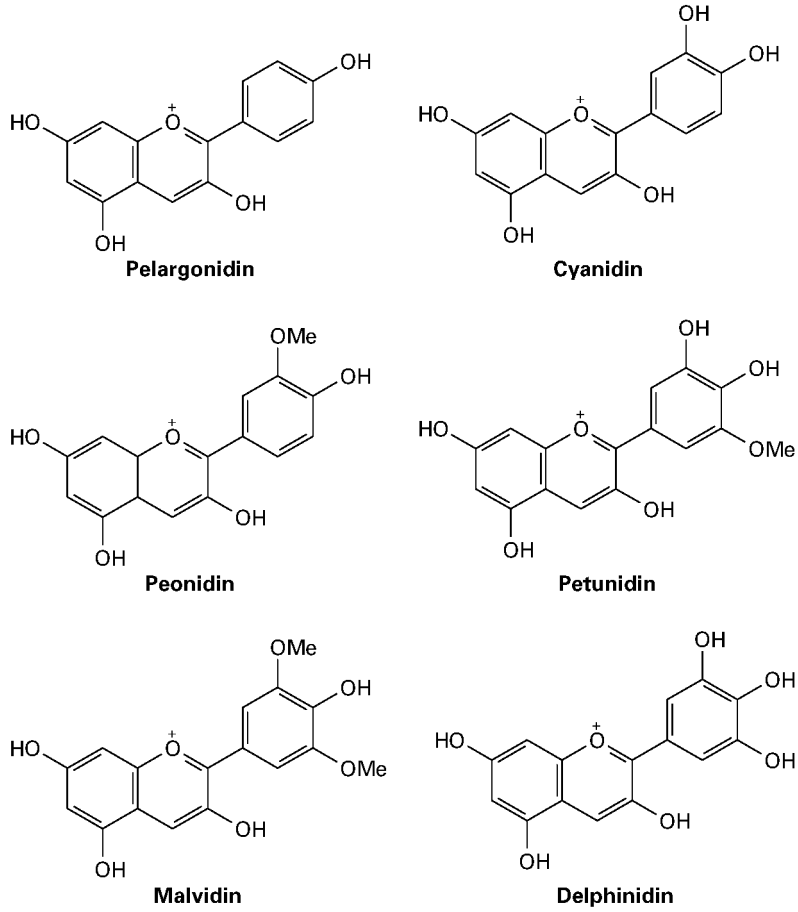


Figure 6 Structures of the most usual anthocyanidins.

Table 5 Thin-layer chromatography of anthocyanidins and anthocyanins (R_F values)

Pigments	*Source*	*Type*	*System*		
			1	2	3
Anthocyanidins					
Delphinidin	a			0.11	0.03
Petunidin	a			0.20	0.05
Cyanidin	a			0.22	0.06
Malvidin	a			0.27	0.07
Peonidin	a			0.31	0.08
Pelargonidin	a			0.35	0.11
Anthocyanins					
Dp-3-glu	b	Mono	0.08	0.38	0.13
Pt-3-glu	c	Mono	0.13	0.49	0.23
Cy-3-glu	b	Mono	0.17	0.51	0.25
Mv-3-glu	c	Mono	0.22	0.64	0.34
Pn-3-glu	c	Mono	0.25	0.64	0.38
Pg-3-glu	d	Mono	0.32	0.65	0.40
Dp-3-rut	b	Bioside	0.24	0.69	0.36
Cy-3-rut	b	Bioside	0.35	0.69	0.49
Pn-3-rut	e	Bioside	0.47	0.76	0.63
Cy-3-sam	f	Bioside	0.47		0.64
Cy-3-sop	f	Bioside	0.62	0.81	0.75
Cy-3,5-diglu	g	di	0.38	0.70	0.52
Pn-3,5-diglu	g	di	0.49	0.81	0.67
Cy-3-glurut	f	tri	0.80	0.86	0.88

Pigment name abbreviations: Dp, Delphinidin; Pt, Petunidin; Cy, Cyanidin; Mv, Malvidin; Pn, Peonidin; Pg, Pelargonidin; glu, glucoside; rut, rutinoside; sam, sambubioside; sop, sophoroside; glurut, (2^G-glucosyl) rutinoside.
Sources: a, hydrolysis product; b, *Ribes nigrum* berry; c, *Vitis vinifera* fruit; d, *Fragaria* spp. berry; e, *Prunus* spp. fruit; f, *Rubus idaeus* berry; g, *Fuchsia* spp. flowers. Glycoside types are indicated as mono (monoglycoside), di (diglycoside), tri (triglycoside) and bioside (glycosylglycoside). R_F values are given for cellulose layers (0.1 mm, Merck Art. 5716), using for development mixtures of concentrated hydrochloric acid, formic acid and water as follows: system 1 (19 : 19 : 62), system 2 (7 : 51 : 42) and system 3 (25 : 24 : 51).

example when the sugar moiety is acylated, this may be replaced by methanol containing 5% acetic acid. After concentration, the solution can be directly subjected to thin-layer chromatography on cellulose.

The individual anthocyanins may be subjected to hydrolysis by refluxing in hydrochloric acid for an hour, after which time the liberated anthocyanidins are extracted into 1-pentanol. The solution is evaporated to dryness under a stream of nitrogen, then redissolved in methanolic hydrochloric acid. This solution can be used directly for chromatography.

Results obtained for the separation of anthocyanins and anthocyanidins on cellulose layers using hydrochloric acid–formic acid–water mixtures as developing solvents are to be found in **Table 5**. The major trends in the results are immediately obvious. Looking first at the anthocyanidins it can be seen that polarity increases with the number of hydroxyls present, while the presence of methoxyls has a much smaller effect. Examination of the monoglucosides shows that while a considerable increase in polarity is observed as a result of glucosylation, the effect seen above is carried over to this group of compounds. The same trend is seen in the even more polar biosides where the rutinosides are ordered in the same way and in the diglucosides where cyanidin diglucoside is less polar than the corresponding peonidin derivative. The single trigylcoside examined is, as expected, even more polar than the diglycosides. These general findings are well seen in the chromatographic results obtained on separation of the pigments from raspberry and blackcurrant fruits (**Figure 7**).

Extraction procedure The intact tissue is cut into small pieces and then macerated with 3 volumes methanol containing 1% concentrated hydrochloric acid for 3 min. The extract is decanted and retained, and the procedure repeated twice. The extracts are then collected together and an equal volume of petroleum ether (40–60°C) added. After shaking vigorously for 2 min the layers are separated and the aqueous extract re-extracted in the same way with a further portion of petroleum ether. The defatted extract is then dried under reduced pressure. The pigments are redissolved in methanolic hydrochloric acid for chromatographic analysis.

DiroBooks

Hydrolysis The defatted extract is dissolved in equal volumes of methanol and 8 mol L^{-1} hydrochloric acid and refluxed for 1 h. The mixture is then cooled and the free anthocyanidins extracted by shaking with 1-pentanol. The 1-pentanol layer is separated and the solvent is removed in a stream of nitrogen (35°C). The anthocyanidins are then redissolved in methanol containing 1% concentrated hydrochloric acid. The sugars freed during this process remain in the water solution and these too may be analysed by thin-layer methods.

Identification Anthocyanins are not readily available commercially and are best extracted freshly from established sources. The anthocyaninidins are then readily obtained by hydrolysis as described above. The specialized flavonoid monographs suggested for further reading give long lists of suitable sources.

Other Pigments

A large number of other pigment groups occur in nature. Among the less polar and more abundant

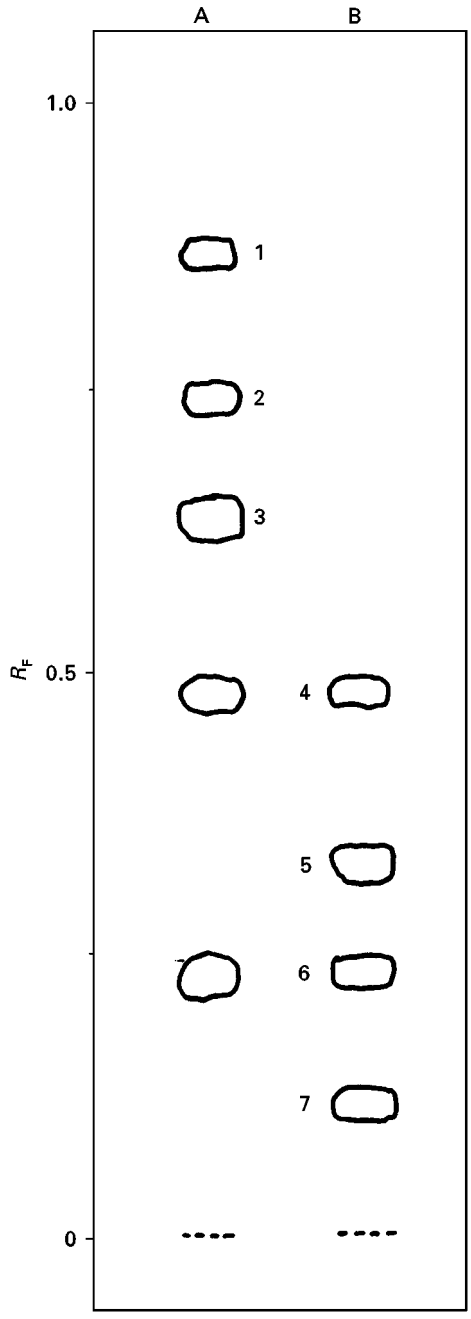


Figure 7 Chromatography of anthocyanins from (A) raspberry (*Rubus idaeus*) and (B) blackcurrant (*Ribes nigrum*). Cellulose layer (0.1 mm, Merck Art. 5716) using for development a mixture of concentrated hydrochloric acid, formic acid and water (25 : 24 : 51). Identification of zones: 1, cyanidin-3-(2^G-glucosyl)rutinoside; 2, cyanidin-3-sophoroside; 3, cyanidin-3-sambubioside; 4, cyanidin-3-rutinoside; 5, delphinidin-3-rutinoside; 6, cyanidin-3-glucoside; 7, delphinidin-3-glucoside.

of these are the various quinones, which can be investigated by thin-layer chromatography, although no particular system seems to be useful for more than a small groups of compounds. Other compounds which should be mentioned are the many groups of tetrapyrrole pigments, both cyclic and acyclic, but these are highly polar and individual chromatographic systems are again required for each type.

Future Trends

The popularity of thin-layer chromatography for the analysis of pigments seems likely to continue as interest in natural pigments as food colorants increases. The possibility of carrying out some 20 parallel analyses on a single plate in 1 h ensures that the method represents a good alternative to high performance liquid chromatography. The fact that thin-layer chromatographic systems are cheap and easy to use, that the results are immediately observable, and that the method is both rugged and transportable will probably lead to increasing applications in the production side of the food industry. Applications of this type would be expected to lead fairly rapidly to an increase in the relatively small number of specialized spray reagents presently available for work with pigments.

Further Reading

Andersen ØM and Francis GW (1996) Natural products. In: Sherma J and Fried B (eds) *Handbook of Thin Layer Chromatography*, pp. 715–752. New York: Marcel Dekker.

Britton G, Liaaen-Jensen S and Pfander H (eds) (1995) *Carotenoids*, vol. 1A. *Isolation and Analysis*. Basel: Birkhauser.

Goodwin TW (ed.) (1976) *Chemistry and Biochemistry of Plant Pigments*, 2nd edn. London: Academic Press.

Goodwin TW (1980) *The Biochemistry of the Carotenoids I–Plants*. London: Chapman & Hall.

Goodwin TW (1984) *The Biochemistry of the Carotenoids II–Animals*. London: Chapman & Hall.

Gross J (1991) *Pigments in Vegetables*. New York: Van Nostrand Reinhold.

Harborne JB (1967) *Comparative Biochemistry of the Flavonoids*. London: Academic Press.

Harborne JB and Mabry TJ (eds) (1982) *The Flavonoids: Advances in Research*. London: Chapman & Hall.

Harbone JB, Mabry TJ and Mabry H (eds) (1975) *The Flavonoids*. London: Chapman & Hall.

Markakis P (1982) *Anthocyanins as Food Colours*. London: Academic Press.

Markham KR (1982) *Techniques of Flavonoid Identification*. London: Academic Press.

Parker L (ed.) (1992) *Methods in Enzymology*, vol. 213 – *Carotenoids*. San Diego: Academic Press.

Sestàk Z (1982) Thin layer chromatography of chlorophylls 2. *Photosynthetica* 16: 568–617.

Stahl E (1969) *Thin Layer Chromatography*, 2nd edn. New York: Springer Verlag.